



저작자표시-비영리-동일조건변경허락 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.
- 이차적 저작물을 작성할 수 있습니다.

다음과 같은 조건을 따라야 합니다:



저작자표시. 귀하는 원저작자를 표시하여야 합니다.



비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.



동일조건변경허락. 귀하가 이 저작물을 개작, 변형 또는 가공했을 경우에는, 이 저작물과 동일한 이용허락조건하에서만 배포할 수 있습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 [이용허락규약\(Legal Code\)](#)을 이해하기 쉽게 요약한 것입니다.

[Disclaimer](#)

A THESIS
FOR THE DEGREE OF MASTER OF SCIENCE

**Establishment of *Bacillus thuringiensis* based exogenous double-
stranded RNA production platform**

Bacillus thuringiensis 기반의 외인성 이중가닥 RNA 생산
플랫폼 구축

By
Min Gu Park

Major in Entomology
Department of Agricultural Biotechnology
Seoul National University
February, 2018

**Establishment of *Bacillus thuringiensis* based exogenous double-
stranded RNA production platform**

Bacillus thuringiensis 기반의 외인성 이중가닥 RNA 생산
플랫폼 구축

**UNDER THE DIRECTION OF ADVISER YEON HO JE
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF
SEOUL NATIONAL UNIVERSITY**

**By
Min Gu Park**

**Major in Entomology
Department of Agricultural Biotechnology
Seoul National University
February, 2018**

**APPROVED AS A QUALIFIED THESIS OF Min Gu Park
FOR THE DEGREE OF MASTER OF SCIENCE
BY THE COMMITTEE MEMBERS**

CHAIRMAN Kwang Pum Lee _____

VICE CHAIRMAN Yeon Ho Je _____

MEMBER Si Hyeock Lee _____

Establishment of *Bacillus thuringiensis* based exogenous double-stranded RNA production platform

Major in Entomology

Department of Agricultural Biotechnology

Seoul National University

February 2018

Min Gu Park

ABSTRACT

RNA interference (RNAi) is a post-transcriptional gene regulation mechanism found in virtually all plants and animals including insects. RNAi has been considered as an alternative strategy to control agricultural pests whereby double-stranded RNA (dsRNA) triggers a potent and specific inhibition of its homologous mRNA. It also can be as potential therapeutic strategy for treating and preventing of diseases in beneficial insects for crops. Since small dsRNAs are required for various RNAi applications, there is a need for cost-effective methods for producing large quantities

of high-quality dsRNA. In this study, to produce exogenous dsRNA through simple and cost-effective methods, there is a focus on developing entomopathogenic bacteria *Bacillus thuringiensis*.

The first step was to find a target that confirming dsRNA triggered gene silencing phenomenon such as RNAi system in Sacbrood virus (SBV). It is a classified as the order Picornavirales, family Iflaviridae, genus Iflavirus with a positive single-stranded RNA genome, is one of the most fatal emerging honeybee virus that cause a serious threat to the Asian honeybee *Apis cerana*, and tends to cause bee colony and even the whole apiary collapse. In this research, dsRNAs targeting SBV structural protein were synthesized to control SBV. These dsVP1, dsVP3, and dsRdRp took RNAi effects in the SBV infected *Apis cerana* workers, as fed with these dsRNAs and confirmed that among the three dsRNAs, *vp1* gene specific dsRNA (dsVP1) showed a powerful effect of reducing SBV replication.

For the second step, it was necessary to use the characteristics of *Bacillus thuringiensis*. The gram-positive bacterium *Bacillus thuringiensis* has insecticidal proteins produced during the stationary/sporulation phase of growth with expression of their encoding genes driven by sporulation dependent promoters. To develop a dsRNA mass-production platform utilizing *Bacillus thuringiensis*, the pHT1K-SBV *vp1* and pHT1K-EGPF plasmid vectors which transcribe sense and anti-sense target gene under the control of *cytIAa* sporulation-dependent promoter with STAB-SD sequence were constructed. According to the confirmation of target gene expression by reverse transcription PCR, relative transcription level by qPCR analysis, and dsRNA quantification by hybridization analysis, it suggested that a potential of

***Bacillus thuringiensis* becoming a new platform in dsRNA production.**

Finally, to confirm if these dsRNAs play their role in RNAi system properly, the bioassay using SBV infected *Apis cerana* was conducted. Ingestion of these dsRNAs derived from transformed *Bacillus thuringiensis* to SBV infected Asian honeybee, *Apis cerana* showed powerful effect on SBV suppression. As a result, it was confirmed that the dsRNAs derived from *B.thuringiensis* play their role in RNAi system.

Key words: *Bacillus thuringiensis*, double-stranded RNA, RNA interference, *Apis cerana*, Sacbrood virus

Student Number; 2016-21725

TABLE OF CONTENTS

ABSTRACT	II
TABLE OF CONTENTS	V
LIST OF TABLES.....	VIII
LIST OF FIGURES.....	IX
INTRODUCTION	1
LITERATURE REVIEW	4
1. RNA interference (RNAi)	4
2. Sacbrood virus (SBV)	5
3. General characteristics of <i>Bacillus thuringiensis</i>	7
MATERIAL AND METHODS	10
1. Target gene design from SBV genes	10
2. Synthesis of target gene dsRNA	10
3. Honeybees and Sacbrood virus	12
4. <i>Apis cerana</i> bioassay.....	12
5. Detection of SBV gene knockdown.....	15
6. <i>Bacillus thuringiensis</i> strain, growth condition	17

7.	Plasmid vector construction of pHT1K-SBV <i>vp1</i> and EGFP	19
8.	<i>Bacillus thuringiensis</i> transformation	19
9.	Total RNA extraction from <i>B. thuringiensis</i>	20
10.	Transformed <i>Bacillus thuringiensis</i> RT-PCR and qPCR analysis	21
11.	Hybridization analysis	23
12.	Optimization of <i>B.thuringiensis</i> autolysis conditions for bioassay	23
13.	Apis cerana bioassay with pHT1K-SBV <i>vp1</i>	24
RESULTS		26
1.	Target gene selection and confirmation of dsRNA	26
2.	Detection of SBV gene knockdown by feeding SBV specific dsRNA	28
3.	Plasmid vector construction of pHT1K-SBV <i>vp1</i> and EGFP	31
4.	Confirmation of transformed <i>B. thuringiensis</i> dsRNA	38
A.	Confirmation of dsRNA by RT-PCR	38
B.	Confirmation of dsRNA by qPCR	38
C.	Confirmation of dsRNA by hybridization analysis.....	40

5.	Optimization of <i>B. thuringiensis</i> autolysis conditions for bioassay ..	42
6.	A. cerana bioassay for transformed <i>B. thuringiensis</i> induced RNA interference	44
A.	Concentration dependent honeybee safety test.	44
B.	Detection of SBV gene knockdown by feeding of dsRNA from pHT1K-SBV <i>vp1</i> transformed <i>B. thuringiensis</i>	44
C.	Concentration dependent suppression of SBV by dsRNA from pHT1K-SBV <i>vp1</i> transformed <i>B.thuringiensis</i>	45
	DISCUSSION.....	48
	LITERATURES CITED.....	51
	ABSTRACT ON KOREAN.....	56

LIST OF TABLES

Table 1. List of primers used for dsRNA preparation	11
Table 2. List of treatments and concentration used for <i>Apis cerana</i> bioassay.	14
Table 3. List of primers used for quantitative real-time PCR	16
Table 4. Composition of culture media for a strain of <i>B.thuringiensis</i>	18
Table 5. List of primers used for reverse transcription PCR and quantitative real-time PCR	22

LIST OF FIGURES

Fig. 1. Sacbrood virus genome structure and siRNAs candidates for RNA interference target gene design..	27
Fig. 2. Synthesis of SBV VP1, VP3, and RdRp dsRNAs by using in vitro transcription system.....	27
Fig. 3. The relative virus replication levels of dsRNA treated workers.....	29
Fig. 4. The relative virus replication levels of synergistic effect of dsRNA.	30
Fig. 5. construction map of pHT1K-SBV <i>vp1</i> and pHT1K-EGFP cassette.....	32
Fig. 6. Nucleotide sequences of pHT1K-SBV <i>vp1</i> vector cassette.....	34
Fig. 7. Nucleotide sequences of pHT1K-EGFP vector cassette.	36
Fig. 8. Construction map of vectors.	37
Fig. 9. Confirmation of dsRNA; RT-PCR & qPCR analysis.....	39
Fig. 10. Hybridization analysis result.	41
Fig. 11. Optimization of autolysis conditions for bioassay.....	43
Fig. 12. pHT1K-SBV <i>vp1</i> vector transformed <i>Bacillus thuringiensis</i> total RNA concentration dependent honeybee safety test.	46
Fig. 13. <i>Apis cerana</i> bioassay with total RNA of transformed <i>Bacillus thuringiensis</i>.	47

INTRODUCTION

RNA interference (RNAi) is a post-transcriptional gene regulation mechanism found in virtually all plants and animals including insects. It is triggered by double-stranded RNA (dsRNA) precursors that vary in length and origin. These dsRNAs are processed into short RNA duplexes of 21 to 25 nucleotides in length, which then guide the recognition and ultimately the cleavage or translational repression of complementary single-stranded RNAs, such as mRNAs or viral genomic RNAs. RNAi has been considered as an alternative strategy to control agricultural pests whereby dsRNA triggers a potent and specific inhibition of its homologous mRNA. It is also applied to the suppression of virus replication.

The Asian honeybee, *Apis cerana* is a native honeybee species in Korea and has been noted for its strong resistance to the ectoparasitic mites (Peng et al., 1987) such as *Varroa destructor* which causes significant damage to European honeybee, *Apis mellifera* worldwide (Rosenkranz et al., 2010). However, since 2009, *A. cerana* in Korea has been endangered because of the outbreak of sacbrood virus (SBV) disease which caused over 75% of serious colony loss, as a result SBV is a major threat to the honeybee industry in Korea. SBV, which is classified into the order Picornavirales, family Iflaviridae, genus Iflavirus with a positive single-stranded RNA genome, is one of the most fatal emerging honeybee viruses that cause a serious threat to *A. cerana*, and tends to cause bee colony and even the whole apiary collapse. The larvae infected with SBV show specific symptoms such as accumulation of virus enriched ecdysal fluid beneath their unshed skin, and failure of pupation causing larval death (Bailey et al., 1964). The infected larvae are often removed

alive from the beehive by worker bees even before death, and the highly epidemic property of SBV can result in a rapid collapse of colonies in wide area where the bees share the same source of flower nectar and pollen. Although SBV causes a fatal infection in bee larvae, it is also infectious to adult bees. However, in this case obvious physical signs of disease are lacking (Bailey, 1969) while the infected workers may have a decreased life-span (Bailey and Fernando, 1972). SBV occurs most frequently in spring and this is believed to reflect the availability of susceptible larvae and young adults which is greatest in those periods when the colony grows most rapidly (Bailey, 1969).

To control SBV, dsRNA targeting SBV VP1 structural protein was synthesized. Since small dsRNAs are required for various RNAi applications, there is a need for cost-effective methods for producing large quantities of high-quality dsRNA. To produce exogenous dsRNA through simple and cost-effective methods, there is a focus on developing entomopathogenic bacteria *Bacillus thuringiensis*.

The gram-positive bacterium *B. thuringiensis* has insecticidal proteins produced during the stationary/sporulation phase of growth with expression of their encoding genes driven by sporulation dependent promoters. The stability of mRNA is an important contributor to the high level of toxin production in *B. thuringiensis*. The half-life of *cry* mRNA, about 10 min, is at least five-fold greater than that of an average bacterial mRNA (Glatron and Rapoport, 1972). Furthermore, Shine-Dalgarno sequence (GAAAGGAGG) which has been designated STAB-SD, mapping increase mRNA stability. The binding of a 30S ribosomal subunit to this STAB-SD sequence may protect the mRNA against ribonuclease activity, resulting in a stable transcription (Agaisse and Lereclus, 1996).

In this study, it was focused on developing dsRNA mass-production platform utilizing

B. thuringiensis, the pHT1K-SBV vp1 and pHT1K-EGFP plasmids vector which transcribe sense and anti-sense target gene under the control of *cyt1Aa* sporulation-dependent promoter with STAB-SD sequence were constructed. The transcription of dsRNAs were confirmed and quantified by qPCR and hybridization analysis. Additionally, to confirm if this dsRNA plays its role in RNAi system properly, the SBV infected *A. cerana* bioassay was conducted.

The method allows for several dsRNAs to be produced in parallel at much reduced cost and produces effective dsRNAs. This method is also useful to produce long RNA fragments of sequence that may be difficult to obtain by chemical synthesis. This method is suited to production of RNAs of specific sequences at large scale or to the production of libraries of RNA molecules of varying length and sequence which would otherwise be prohibitively expensive by chemical synthesis.

LITERATURE REVIEW

1. RNA interference (RNAi)

Gene silencing by RNAi is a powerful technology for manipulating gene expression and can serve as a novel approach for controlling agriculture pests and potential therapeutic strategy for treating human diseases. Furthermore, it is also for the treatment and prevention of diseases in beneficial insects for crops.

In 1998, Fire et al. (Fire et al., 1998) described a process in which the application of exogenous dsRNA silenced the homologous endogenous mRNA in the *Caenorhabditis elegans* and called RNA interference (RNAi). Although new for animals, the technique was already described as ‘post-transcriptional gene silencing’ in plants. Moreover, those techniques appeared to be remarkably well conserved in several eukaryotes. RNAi technique soon proved to be promising in several research fields: in medicine to control cancers and viral disease and in molecular biology for gene function determination and gene knockdown in eukaryotes.

The mechanism of RNAi is triggered by dsRNA precursors that vary in length and origin. These dsRNAs are processed into small RNA, 21 to 28 nucleotides in length that mimic products of Dicer-processed dsRNA and can be incorporated into the RNA-induced silencing complex (RISC) to trigger the degradation of mRNA targets. For this reason, artificial introduction of long dsRNAs has been adopted as a tool to inactivate gene expression, both in cultured cells and in living organisms.

RNA silencing mechanisms were first recognized as antiviral mechanisms that protect organisms from RNA viruses (Waterhouse et al., 2001). The single-stranded RNA viruses generate dsRNA intermediates during their replication cycle and likely have significant secondary RNA structure within their genomes (Miranda et al., 2013), either of which may serve as Dicer-like substrates and trigger the honeybee small-interfering (siRNA) pathway. For example, the role of RNAi in honey bee antiviral defense was first demonstrated when bees fed Israeli acute paralysis virus (IAPV) and IAPV-specific dsRNA had reduced IAPV levels as compared to bees fed only virus (Maori et al., 2009). Additionally, IAPV-specific siRNAs were detected by hybridization analysis in the IAPV-specific dsRNA treated bees. It is suggested that Dicer-like endoribonuclease cleaved the dsRNA and virus genomes. Likewise, when Deformed wing virus specific dsRNA were fed to larvae and adult bees, it had reduced mortality, virus load, and deformed wing symptoms (Desai et al., 2012).

2. Sacbrood virus (SBV)

The importance of honeybees to the global world economy includes their contribution to the honey production and the pollination of a various major fruit of crops.

However, not much different from other social insects, honeybees are usually threatened by a various number of pathogens from bacteria, fungi and parasites to protozoa, and viruses (Consortium, 2006; Schmid-Hempel and Schmid-Hempel, 1998), because of their crowded and warm living conditions, and in social interactions, such as mutual grooming and food sharing exchange. Among them, over 18 honeybee viruses have been identified (Allen and Ball, 1996).

The Asian honeybee, *A. cerana* is a native honeybee species in Korea and has been noted

for its strong resistance to the ectoparasitic mites (Peng et al., 1987) such as *Varroa destructor* which causes significant damage to *A. mellifera* worldwide (Rosenkranz et al., 2010). However, since 2009, *A. cerana* in Korea has been endangered because of the outbreak of SBV disease which caused over 75% of serious colony loss, as a result SBV is a major threat to the honeybee industry in Korea.

SBV is classified as the order Picornavirales, family Iflaviridae, genus Iflavirus with a positive single-stranded RNA genome (Lanzi et al., 2006). The complete genome of SBV was also sequence which includes 8832 nucleotides encoding 2858 amino acids (Choe et al., 2012).

The larvae infected with SBV show specific symptoms such as accumulation of virus enriched ecdysal fluid beneath their unshed skin, and failure of pupation causing larval death (Bailey et al., 1964). The infected larvae are often removed alive from the beehive by worker bees even before death, and the highly epidemic property of SBV can result in a rapid collapse of colonies in wide area where the bees share the same source of flower nectar and pollen. Although SBV causes a fatal infection in bee larvae, it is also infectious to adult bees. However, in this case obvious physical signs of disease are lacking (Bailey, 1969) but such infected workers may have a decreased life-span (Bailey and Fernando, 1972). Sacbrood virus occurs most frequently in the spring and this is believed to reflect the availability of susceptible larvae and young adults which is greatest in those periods when the colony grows most rapidly (Bailey, 1969).

Different methods have been introduced in attempts to control SBV in *A. cerana*, including the selection of resistant bee populations, clearance of the infected hives, prevention of the temperature fluctuation in the hives, enough food supply, and use of some

herbs, however, no effective results are obtained. RNAi or post-transcriptional gene silencing (PTGS) is the phenomenon whereby dsRNA blocks the expression of its homologous gene, and has been reported in prokaryotes, nematodes, and other invertebrate animals (Geley and Müller, 2004; Hannon, 2002). Recently, IAPV, a bee affecting virus associated with colony collapse disorder was found to be silenced by dsRNA ingestion (Maori et al., 2009).

3. General characteristics of *Bacillus thuringiensis*

B. thuringiensis is belonging to the Bacillaceae family and closely related to *Bacillus cereus*. *B. thuringiensis* is a ubiquitous, spore-forming, Gram-positive bacterium which can be readily isolated from a variety of environmental sources including soil, water, plant surfaces, grain dust, dead insects, and insect feces was characterized by its ability to produce a parasporal protein crystal during sporulation. The first remarkable characteristics in *B. thuringiensis* life cycle is when nutrients and environmental conditions are sufficient, the spore germinates producing a vegetative cell that grows and reproduces by binary fission. Cells continue to multiply until one or more nutrients, such as sugars, amino acids, or oxygen, become insufficient for continued vegetative growth. Under those conditions, *B. thuringiensis* sporulates producing a spore and parasporal body, and then composes primarily of one or more insecticidal proteins in the form of crystalline inclusions (A Federici et al., 2010). These proteins are highly toxic to a wide variety of important agricultural and health-related insect pests. As their high specificity and their safety for the environment, crystal proteins are a valuable alternative to chemical pesticides for control of insect pests in agriculture. Insecticidal crystal proteins from *B. thuringiensis* have been

used intensively as biopesticides for the several decades. It has been proposed that the rational use of *B. thuringiensis* toxins will provide a variety of alternatives for insect control and for overcoming with the problem of insect resistance to pesticides.

Insecticidal proteins are typically produced during sporulation, with the expression of their encoding genes being driven by one or two sporulation dependent promoters (Wong et al., 1983). Transcription from these promoters, referred to as BtI, and BtII, is initiated by RNA polymerase complexes that contain, sigma-E and sigma-K like factors, respectively (Agaisse and Lereclus, 1995; Baum and Malvar, 1995; Wong et al., 1983). Individual Cry proteins are toxic to insects and classified as CryI, CryII, CryIII, CryIV, etc., according to their activity spectra (Höfte and Whiteley, 1989). These insecticidal activities are usually restricted to a species within particular order of lepidoptera, diptera, coleoptera, and hymenoptera. The most common endotoxin proteins that produced in *B. thuringiensis* are those of the CryI class. These are 130- to 135-kDa protoxins, which form a single large bipyramidal crystal about the size of a spore in each cell.

B. thuringiensis, *B. cereus* (sensu stricto), and *Bacillus anthracis*, called the *B. cereus* group, form a highly homogeneous subdivision of the genus *Bacillus* and are often regarded as a single species due to their high genetic relatedness (Rasko et al., 2005). In these species *B. cereus* group, only *B. thuringiensis* has been recognized as being safe to humans and animals. Most *B. thuringiensis* strains harbor diverse plasmids, while a plasmidless strain may serve as an indispensable tool for the genetic engineering of *B. thuringiensis*. Therefore, *B. thuringiensis* serovar israelensis 4Q7, which is an acrySTALLIFEROUS, plasmidless *B. thuringiensis* strain widely used as a recombination host.

In *Bacillus* species, the endospore develops in a sporangium consisting of two

cellular compartments known as the mother cell and the forespore. At the transcriptional level, the development of sporulation is controlled by the successive activation of sigma factors, which bind the core RNA polymerase to direct the transcription from sporulation-specific promoters (Charles P. Moran, 1993).

The stability of mRNA is an important contributor to the high level of toxin production in *B. thuringiensis*. The half-life of *cry* mRNA, about 10 min, is at least fivefold greater than the half-life of an average bacterial mRNA (Glatron and Rapoport, 1972). Furthermore, Shine-Dalgarno sequence (GAAAGGAGG) which has been designated STAB-SD, mapping increase mRNA stability. The binding of a 30S ribosomal subunit to this STAB-SD sequence may protect the mRNA against ribonuclease activity, resulting in a stable transcription (Agaisse and Lereclus, 1996).

MATERIAL AND METHODS

1. Target gene design from SBV genes

The nucleotide sequence of target genes vp1, vp3, RdRp those which belong to SBV were obtained from the RNA-sequencing. The target sequences were applied to the Invitrogen BLOCK-It RNAi Designer software to find the candidate siRNA sites. Each dsRNA for the target gene was designed to include at least five putative siRNA sites.

2. Synthesis of target gene dsRNA

Single-strand cDNA of each target gene was synthesized from total RNA of the SBV-infected larvae of *A. cerana* using the QuantiTect Reverse Transcription Kit (QIAZEN, Germany) according to manufacturer's instruction and target genes were amplified with a set of primers including T7 promoter sequence (5'-TAA TAC GAC TCA CTA TAG-3') at 5'-end (Table 1). Using the amplified products as template, dsRNAs for each the target genes were produced by Genolution Pharmaceuticals (Korea), respectively. Each synthesized dsRNA was confirmed on 0.8% LE agarose gel.

Table 1. List of primers used for dsRNA preparation

Primer name	Nucleotide sequence (5' to 3')	Amplicon
T7-SBVvp1-F	T7 promoter sequence + AGATGTGAATGCTTATCCTGATG	635bp
T7-SBVvp1-R	T7 promoter sequence + TCCTCGCATATACACCAAACTT	
T7-SBVRdRp-F	T7 promoter sequence + TGATTCTCAACACTAGTGCAGG	497bp
T7-SBVRdRp-R	T7 promoter sequence + CCTGGACCGAAATTTGAATA	
T7-SBVvp3-F	T7 promoter sequence + TACCATTGAGACTTATGTGTAT	493bp
T7-SBVvp3-R	T7 promoter sequence + CTCCTGATACTAAAGTACCTATAAC	

*T7 promoter sequence = TAATACGACTCACTATAGGG

3. Honeybees and Sacbrood virus

The *A. cerana* hives that used in this experiment were originated from honeybee farm at Cheongwon-gun, Chungcheongbuk-do (province), South Korea and have been kept at Seoul National University, Seoul, Republic of Korea. The beehive showed no visual sign of disease, and newly laid eggs were observed in the hive which indicates that the queen was actively laying eggs.

Sacbrood virus (SBV) infected larvae with typical symptoms were collected from an apiary at Rural Development Administration, Jeonju-si, Jeollabuk-do, Republic of Korea. The presence of SBV was confirmed by RT-PCR and the larvae were ground in 1ml sterile phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 10mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) with a sterile grinder. SBV RNA-dependent-RNA-polymerase (RdRp) gene was amplified to detect copy number of SBV and the SBV concentration in this experiment 1.0×10^9 virus/mL in 40% (w/v) sucrose solution was quantitated by qPCR analysis.

4. *Apis cerana* bioassay

About 20 *A. cerana* worker bees were collected from healthy apiary into small cages and then worker bees were fed 1.0×10^9 viral genome copies/mL in 40% (w/v) sucrose solution *ad libitum* for 12 hours while bees in negative control cage were fed 40% (w/v) sucrose solution only. After 12 hours, worker bees were fed different kinds of dsRNA containing 40% (w/v) sucrose solution for 48 hours and then their total RNA was extracted

from individual worker bees using Qiazol lysis reagent (Qiagen Ltd., Crawley, UK). Furthermore, experiment was conducted to compare the synergistic effect of SBV gene knockdown by combination of different kinds and concentrations of dsRNA. Total of 16 treatment conditions are listed on table 2.

Table 2. List of treatments and concentration used for *Apis cerana* bioassay.

Treatments	Concentration	
Negative Control (Sucrose only)	40% (w/v) sucrose in DEPC treated water	
Positive Control (SBV only)	1.0 *10 ⁹ virus/mL in 40% (w/v) sucrose solution	
Positive Control (dsRNA): dsGFP	20 µg/mL	50 µg/mL
dsVp1		
dsVp3		
dsRdRp		
dsVp1 + dsVp3		
dsVp1 + dsRdRp		
dsVp3 + dsRdRp		

5. Detection of SBV gene knockdown

dsRNA fed *A. cerana* were collected after 48 hours of treatment and their total RNA was extracted from individual worker bees using Qiazol lysis reagent (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions and quantified by spectrophotometry. After RNA purification, cDNA synthesis was performed using 1st Strad cDNA Synthesis System (LeGene BioSciences, USA) with oligo-dT and random hexamer according to the manufacturer's protocol. To determine the relative titer of the SBV in the worker honeybee, qPCR was performed by using EvaGreen 2X qPCR MasterMix (Applied Biological Materials Inc, Canada) and CFX96 real-time PCR detection system (Bio-Rad, USA). 1 µg of total RNA with oligo-dT primers was used for reverse transcription, and 160 bp of SBV RNA dependent RNA polymerase (RdRp) of Korean isolate SBV (GenBank accession# HQ322114), and 151 bp of Actin gene sequences of *A. mellifera* (GenBank accession# AB023025) were used for qPCR. The primers were designed with Primer3 program (Koressaar and Remm, 2007), and the primer sequences are showed in Table 3. The progress of PCR amplification was as follows; 95°C for 10 minutes, followed by 39 cycles of 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. This was followed by a melting curve program of 55 to 95°C with a heating rate of 0.5°C and final cooling at 10°C. The relative titers of SBV in total RNA samples from virus carrying worker bees were calculated by qPCR and $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with Actin gene transcript as a reference. One-way analysis of variance (ANOVA) was used to compare the mean differences of relative quantification in different strains at each time point with a significance level of $P < 0.05$. Samples were collected from three independent experiments.

Table 3. List of primers used for quantitative real-time PCR

Primer name	Nucleotide sequence (5' to 3')	Amplicon
Actin-qF	ATGCCAACACTGTCCTTTCT	151bp
Actin-qR	GACCCACCAATCCATACGGA	
SBV RdRp-qF	ATTCGGTCCAGGGTTAAT	160bp
SBV RdRp-qR	AGGTGACCCACACTTCTGTT	

6. *Bacillus thuringiensis* strain, growth condition

B. thuringiensis strain 4Q7 which is an acrySTALLiferous strain of *B. thuringiensis* sub. *israelensis*, and plasmidless mutant was used in this experiment. *B. thuringiensis* were grown at 30°C with vigorous shaking in SPY medium for plasmid preparation and GYS medium for expression of dsRNA. The LB medium was used as a primary culture of *B. thuringiensis* transformants and in *E.coli* culture for plasmids preparation. Brain heart infusion (BHI, Difco Co., MD, USA) medium was used to culture competent *B. thuringiensis* cells. Media compositions are described in Table 4.

Table 4. Composition of culture media for a strain of *B.thuringiensis*

Medium*	Component	%(g/L)
LB	Tryton	1
	Yeast extract	0.5
	NaCl	1
GYS	Glucose	0.1
	Yeast extract	0.2
	K ₂ HPO ₄	0.05
	(NH ₄) ₂ SO ₄	0.2
	MgSO ₄	0.002
	MnSO ₄	0.005
	CaCl ₂	0.008
SPY	(NH ₄) ₂ SO ₄	0.2
	K ₂ HPO ₄	1.4
	KH ₂ PO ₄	0.6
	Na ₃ C ₆ H ₅ O ₇ ·2H ₂ O	0.1
	MnSO ₄ ·7H ₂ O	0.02
	Glucose	0.1
	Yeast extract	0.1

*LB: Luria-Bertani; GYS: glucose-yeast extract salt medium; SPY: Spizizen medium.

7. Plasmid vector construction of pHT1K-SBV vp1 and EGFP

To develop dsRNA mass-production platform utilizing *B. thuringiensis*, the pHT1K-SBV vp1 and pHT1K-EGFP plasmid vectors which transcribe sense and anti-sense transgenes under the control of *cyt1Aa* sporulation-dependent promoter with STAB-SD sequence was constructed. Firstly, 2443 bp of vector cassette was synthesized which is containing two pairs of *Cyt1Aa* sporulation dependent promoter sequences, STAB-SD sequences, ribosomal binding site (RBS), and terminal sequences are facing each other, and genes of interest sequences are positioned in the middle of the cassette. The dsRNA can be produced in *B. thuringiensis* by transcription with RNA polymerase. After that, this cassette was cloned in between the *SalI* and *SphI* site of pHT1K which is a *E.coli-B.thuringiensis* shuttle vector. The pHT1K vector has the pUC replication sequence and ampicillin resistant gene for amplification and selection in *E. coli*; the *B. thuringiensis* origin and erythromycin resistance gene, for amplification and selection in *B. thuringiensis*.

8. *Bacillus thuringiensis* transformation

Electroporation was performed according to the method of Lereclus et al., (1989), with a slight modification. Firstly, to make *B. thuringiensis* competent cells, the cells were grown to an OD₆₀₀ of 0.7 in 100 ml BHI with shaking at 30°C. The cells were harvested and washed once in 10 ml of cold distilled water. The pellet was then resuspended in 4 ml of cold sterile polyethyleneglycol (PEG) 6000 (40%, w/v). After that, cell aliquots of 0.4 ml were mixed with the plasmid DNA in 0.2 cm electroporation cuvettes (Bio-Rad Co.,

CA, USA) at 4°C. The Bio-Rad Gene Pulser apparatus was set at 25 μ F and 2.5 kV, and the pulse controller was set to 400 Ω . The cuvette was placed in the safety chamber and the pulse was applied once. Following electroporation, the cells were diluted in 2 ml of a pre-warmed BHI medium and incubated with shaking (180 rpm) for 1 hour at 30°C. After this expression period, the cells were then plated on a nutrient agar medium (Difco Co., USA) containing erythromycin (25 μ g/ml).

9. Total RNA extraction from *B. thuringiensis*.

Bacillus thuringiensis was primary cultured in 10 ml of LB medium with shaking at 30°C and grown until the optical density reached 0.7 at 600 nm. After that, 200 μ l of these cultured bacteria was inoculated in 20 ml of GYS medium for secondary culture with shaking at 30°C for 18 h. The bacterial cells were centrifuged at 6,000 \times g for 5 min at 4°C, and the pellets were suspended in 1 ml of Qiazol lysis reagent (Qiagen Ltd., Crawley, UK). Sodium dodecyl sulfate (SDS) was added to a final concentration of 1% (vol/vol). The suspension was sonicated 10 times on ice at 50% duty cycle for 15 s (Branson Sonifier 250, Branson Ultrasonic Corp., Danbury, CT). After samples were incubated at room temperature for 5 min, 200 μ l of chloroform was added. The samples were mixed thoroughly and centrifuged at 12,000 \times g for 15 min. The aqueous phase was transferred to a fresh tube, and 500 μ l of isopropanol was added and inverted softly. After incubation at room temperature for 10 min, RNA was collected by centrifugation at 12,000 \times g for 10 min. The RNA pellets were washed with 1 ml of 75% ethanol, spun at 7,500 \times g for 5

min, dried, and dissolved in 25 µl of diethyl pyrocarbonate-treated double-distilled water. RNA concentrations were determined by measuring the absorption at 260 nm with a spectrophotometer.

10. Transformed *Bacillus thuringiensis* RT-PCR and qPCR analysis

One µg of total RNAs were reverse transcribed to cDNA with the QuantiTect® Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Firstly, to confirm the dsRNA transcription from each strand, cDNA was made by only using forward primer and reverse primer respectively. After that, reverse transcription PCR was performed as described previous PCR conditions. Secondly, to figure out relative transcription level of transformed *B. thuringiensis* transgene, qPCR analysis was performed. The transformed *B. thuringiensis* RNA samples were extracted over different cultivation times of 14, 18, 22, and 26 hours and qPCR were performed by using EvaGreen 2X qPCR MasterMix (Applied Biological Materials Inc, Canada) and CFX96 real-time PCR detection system (Bio-Rad, USA). A spore structure gene (*ssPE*) sequences of *B. thuringiensis* were used as reference. The primers used in reverse transcription PCR and qPCR were showed in Table 5.

Table 5. List of primers used for reverse transcription PCR and quantitative real-time PCR

Primer name	Nucleotide sequence (5' to 3')	Amplicon
SBV vp1-F	CCAGATGTGAATGCTTATCCTGA	602bp
SBV vp1-R	GTACCTCCTCGCATATACACCA	
EGFP-F	ATGGTGAGCAAGGGCGAG	602bp
EGFP-R	TAGTGGTTGTCGGGCAGC	
qSBV vp1-F	ACGGATGAGTGCCAATCTCA	192bp
qSBV vp1-R	TGCTCTAATCCCCGTAGCCT	
qEGFP-F	CGCGACGGCAACTACAAGACCCG	125bp
qEGFP-R	CGCTTGTA CTCCAGCTTGTGCCC	
ssPE-F	GAGAAAGATGAGTAAAAACAACAA	188bp
ssPE-R	CATTTGTGCTTTGAATGCTAG	

11. Hybridization analysis

To confirm the production of dsRNA of transgene, hybridization assay to total RNA of *B. thuringiensis* was performed according to the manufacturer's instruction (DIG luminescent detection kit, Roche). Total RNA from bacterial cells in stationary phase before autolysis begin, was extracted using the Qiazol lysis reagent (Qiagen Ltd., Crawley, UK). Total RNAs (5, 10, 20 µg) from *B. thuringiensis* 4Q7 strain and pHT1K-EGFP transformant *B. thuringiensis* was loaded onto 0.8% LE agarose gels, electrophoresed and transferred to positive charged nylon membranes in 20X SSC buffer followed by UV crosslinking to fix the RNA. For pre-hybridization as well as hybridization, hybridization solution was heated to 68°C and added to the membrane. The membrane was incubated by slow rotation for 2 h at 68°C. Digoxigenin-labeled probes were denatured for 10 min at 98°C and cooled down on ice before addition to preheated hybridization solution. The rest of the process was performed as described in the DIG System Users Guide (Roche Diagnostics).

12. Optimization of *B.thuringiensis* autolysis conditions for bioassay

dsRNA for *A. cerana* bioassay from transformed *B. thuringiensis*, it was harvested by using the characteristics of *B. thuringiensis* autolysis, which is a process of spore release from sporulating cells. The following work was performed to find the optimized *B. thuringiensis* autolysis condition. Primary cultures of a *B. thuringiensis* were grown at 30°C in 100 ml flasks, each containing 20 ml of LB medium, until OD₆₀₀ reached 0.7 and 1 ml of primary cultured cells were spread to nutrient agar medium (petri dish 150 × 20

mm SPL Life Sciences) for secondary culture. Cells were grown about 20 h at 30°C, until before the spores released into the medium measured by direct phase contrast microscope. The sporulating cells were harvested by cell scraper (SPL Life Science) and transferred 2 ml of Eppendorf tube. Each tubes contain 1 g of *B.thuringiensis* cells per 500 µl of each buffer. The buffers are NaCl, PBS (phosphate buffered saline), and 2M MgCl₂. The mixtures were resuspended and incubated for 48 hours in each temperature of 4°C, 10°C, 25°C, and 37°C. Totally 12 treatments were set.

13. *Apis cerana* bioassay with pHT1K-SBV vp1

Primary cultures of a pHT1K-SBV vp1 transformed *Bacillus thuringiensis* were grown at 30°C in 100 ml flasks, each containing 20 ml of LB medium, until OD₆₀₀ reached 0.7 and 1 ml of primary cultured cells were spread to nutrient agar medium (petri dish 150 × 20 mm SPL Life Sciences) containing erythromycin for secondary culture. Cells were grown for about 20 hours at 30°C, until before the spores released into the medium observed by phase contrast microscope. The sporulating cells were harvested by cell scraper (SPL Life Science) and transferred to 2 ml Eppendorf tube. Each tube contained 1 g of transformed *B. thuringiensis* cells with 1 g per 500 µl of PBS (phosphate buffered saline). The mixture was resuspended thoroughly and incubated for 48 hours at 37°C. After incubation, the suspensions were centrifuged at 15,000 rpm for 10 min. at 4°C and the supernatant fluid were used for *A. cerana* safety test and bioassay.

Firstly, for the safety test of these samples to honeybees, these samples were mixed in 40% (w/v) sucrose solution in six different concentrations of 0., 0.5, 1.0, 1.5, 3, and 5 µg/µl and fed to honeybee *ad libitum* for ten days, and then checked the mortality (%).

Secondly, to determine the highest concentration with no adverse effect of dsRNA samples to honeybee, feeding of these concentrations of dsRNAs from pHT1K-SBV vp1 transformed *B. thuringiensis* was followed as described in previous and qPCR was conducted.

Third, to confirm the concentration dependent target gene knockdown by dsRNA from pHT1K-SBV vp1 transformed *B.thuringiensis*, honeybees were treated with 0.1, 0.5, 1.0, and 1.5 µg/µl of dsRNA samples and qPCR was conducted.

RESULTS

1. Target gene selection and confirmation of dsRNA

The nucleotide sequence of target genes, vp1, vp3, and RdRp, which belong to SBV were obtained from the RNA-sequencing. The target sequences were applied to the Invitrogen BLOCK-It RNAi Designer software to find the candidate siRNA sites. As a result, including at least five putative siRNA sites, 595, 457, and 453 bp of vp1, vp3, and RdRp genes were selected, respectively (Fig. 1). Ultimately, with T7 promoter sequences, 635, 493, and 497 bp of dsRNAs were synthesized and confirmed in 0.8 % LE agarose gel electrophoresis (Fig. 2).

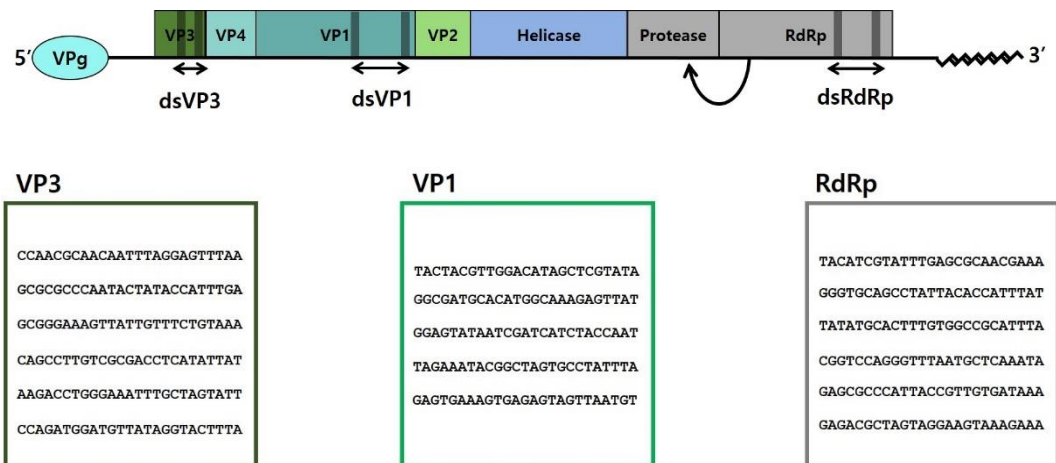


Fig. 1. Sacbrood virus genome structure and siRNAs candidate sequences in the dsRNAs for RNA interference. Each dsRNA for the target gene was designed to include at least five putative siRNA sites.

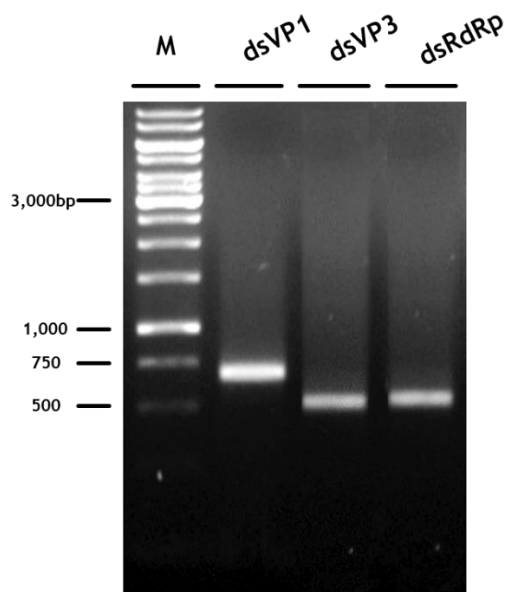


Fig. 2. Synthesis of SBV VP1, VP3, and RdRp dsRNAs by using *in vitro* transcription system. The molecular sizes of each dsRNA are 635, 493, and 497 bp.

2. Detection of SBV gene knockdown by feeding SBV specific dsRNA

Experiments were carried out to examine whether feeding of dsRNAs of SBV sequences would protect bees from subsequent SBV infection. To confirm the target gene knockdown, qPCR was conducted using dsRNA-treated *A. cerana*. In figure 3, it shows the SBV replication after feeding of each dsRNAs. All dsRNAs had reduced SBV levels as compared to the bees fed with virus only. Furthermore, the effect of dsRNA to reduce SBV replication levels was increased according to the relative amount of viral sequences (Fig. 3). At 20 ng/μl concentration, only dsVP1 treated group showed reduced SBV replication levels similar to that of the negative control bees, which were only fed with 40% (w/v) sucrose in DEPC treated water (Fig. 3.A).

There was no significant synergistic effect to reduce SBV levels by using combinations of dsRNA as compared to bees only fed with each of dsRNA respectively (Fig. 4).

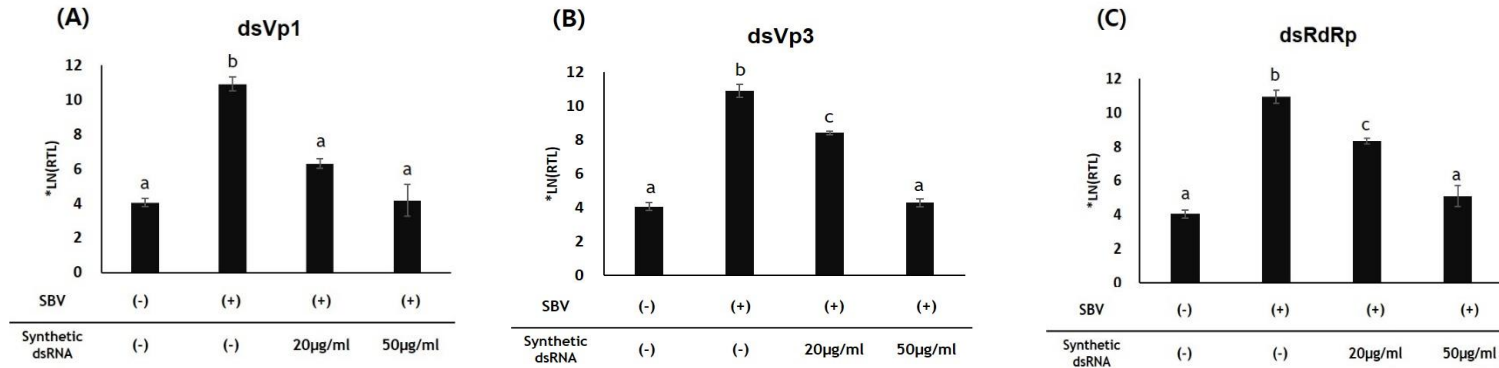


Fig. 3. The relative virus replication levels of dsRNA treated workers. Each sample groups were infected with SBV (10^9 virus genome copies/mL) for 12 hours, and treated with dsRNA (A) SBV-vp1, (B) SBV-vp3, (C) SBV-RdRp for 48 hours followed by total RNA extraction of pooled samples. Y-axis stands for the related transcription level between the actin reference gene and SBV RdRp gene. The relative differential transcription levels were calculated by $\text{Ln}(\Delta\text{Ct})$. Lower number means less transcription. One-way analysis of variance was used to compare the mean differences of relative quantification in different strains at each time point with a significance level of $P < 0.05$.

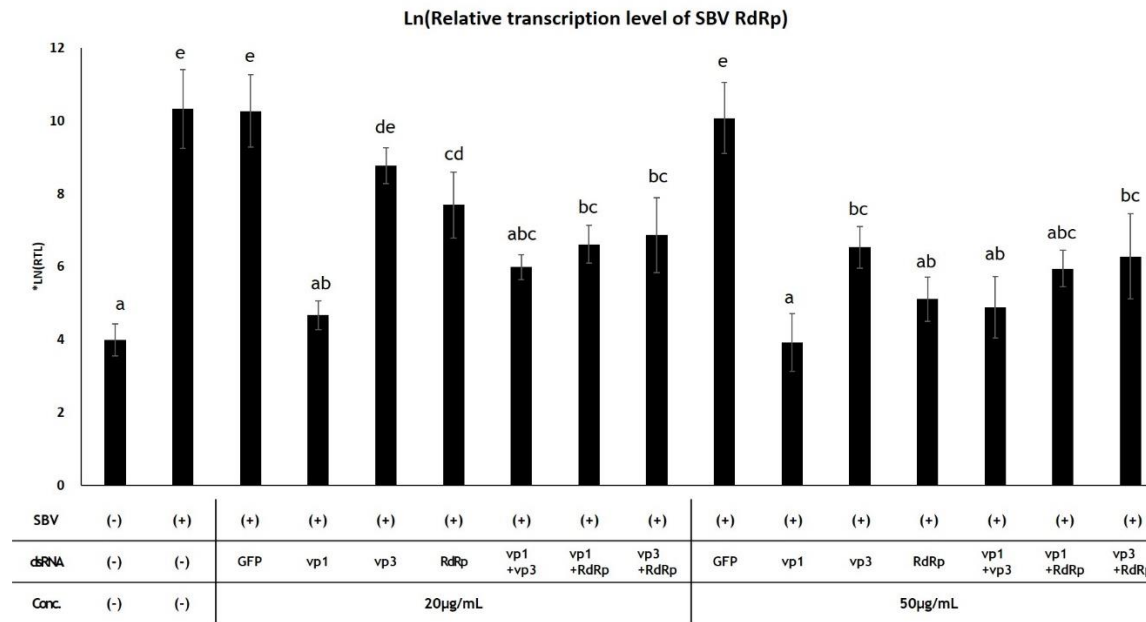


Fig. 4. The relative virus replication levels of dsRNA treated workers including synergistic effect of combination of different kinds and concentration of dsRNA. (*RTL: relative transcription level between the actin reference gene and SBV RdRp gene). One-way analysis of variance was used to compare the mean differences of relative quantification in different strains at each time point with a significance level of $P < 0.05$

3. Plasmid vector construction of pHT1K-SBV vp1 and EGFP

The 2,443 bp length of vector cassette was synthesized which is containing two pairs of *Cyt1Aa* sporulation dependent promoter sequences, STAB-SD sequences, ribosomal binding site (RBS), and terminal sequences which are facing each other and in the middle of the cassette, transgene (SBV vp1, and EGFP) sequences are positioned. The transgene holds on both ends of the *Cyt1Aa* promoters which face each other inward to the transgene in the middle (Fig.5). The nucleotide sequences of 2,443 bp of pHT1K-SBV vp1 vector cassette (Fig. 6), and pHT1K-EGFP vector cassette (Fig. 7) were showed in below. The dsRNA can be produced in *B. thuringiensis* by transcription with RNA polymerase. After that, this cassette was cloned in between the *Sall* and *SphI* site of pHT1K which is *E.coli-B. thuringiensis* shuttle vector. The pHT1K also has the pUC replication sequence and ampicillin resistant gene for amplification and selection in *E. coli*; the Bt origin and erythromycin resistance gene, for amplification and selection in *B. thuringiensis*. Total 7,111 bp of pHT1K-SBV vp1 vector and pHT1K-EGFP vectors were constructed. (Fig. 8).

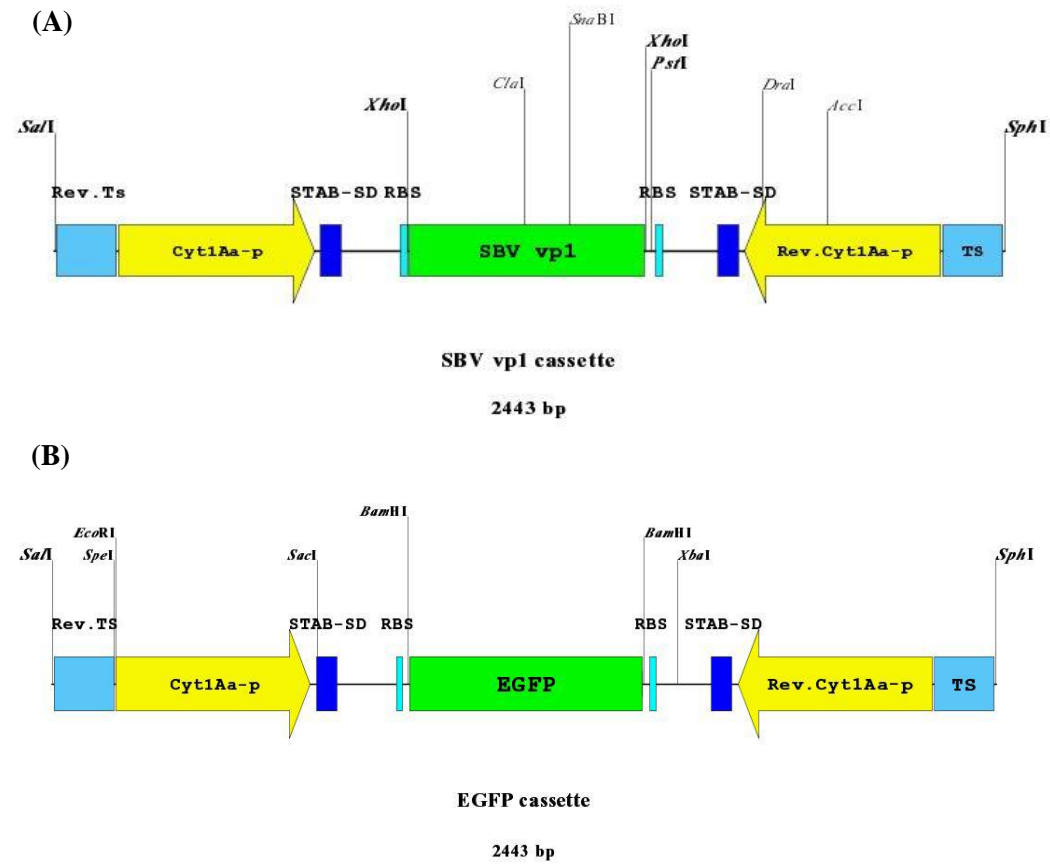


Fig. 5. Structure of (A) pHT1K-SBV vp1 and (B) pHT1K-EGFP cassette. *Cyt1Aa* promoters combined with STAB-SD sequence.

GTCGACTGTATTACTAGAAAATAACATAGTAAAACGGACATCACTCCGTTTCAATGGAGG 60
SaI
 TGATGTCCGTTTTTCATTACAACAAATTACTTATCTATTTGTAATGCTGCTCTTGGACAA 120
Reverse terminal sequence
TTGATTTGAAAACGATATTTAAACCTGAGTTTGCATGAGAACTAGTGAATTCTATTTTCG 180
ATTTCAAATTTTCCAAACTTAAATATGATTGAATGCCTGAGAAAGGTAATAGAGATGTTT 240
TAGTTTATTATGAAGTATTAGGGGCGTCTTTTAAATTCATCTATCAATTTGTGAAATAT 300
ATTACTCAAAACCCAATACCATTCTAAAACCTATTCAAAAATATATATTGCTTTAAAAGAG 360
CATACATACTAAAAAACAGGCATCTTTCGAACTATAGCGCATAGAATACTACGGTGAAT 420
***CytIAa* promoter**
CAAAAACAAATAAAATTTAGGAGGTATATTCAAGTATACAAAAAACTTTAGTGTGAGGG 480
GATTTAGATAAAAAGTATTCGTTATCCTTATAAATTAATTCTTAAACATGCACCAATGTA 540
TACATTAAATAATATTATGTGAATTAAGTCTATCAATTTAATTTATTATGTTACTTTATA 600
TTTGATTAATAATTGCAAGTTTAAATCATAATTTAATGTTGAAAGGCCACTATTCTAAT 660
TAACCTAAGGAGTTGTTTATTTGAGCTCGGTACCCGGGGATAATCTTGAAAGGAGGGATG 720
STAB-SD sequence
CCTAAAAACGAAGAACATTAAAAACATATATTTGCACCGTCTAATGGATTTATGAAAAAT 780
 CATTTTATCAGTTTGAAAATTATGTATTATGATAAGAAAGTCTAGAACGTTATTTAATGA 840
 ACTTTTATAGGTTTTAAATAATATAATGAGAAGTATTTTTTATCAATGATAAGGAGATGAA 900
RBS
 GAAAGCACTCGAGCCAGATGTGAATGCTTATCCTGATGAGCCTCGTACTACGTTGGACAT 960
XhoI
AGCTCGTATATGGGGCTTGAGGAGTACGTTTAATTGGGGATCAGGCGATGCACATGGCAA 1020
AGAGTTATTTAATACCGTTTTGGACCCATGTTTGAGATTTTATGACCAGGATTACGAAGG 1080
ACAAATAACCCCAATGGAATATGTAACGGGTTGTATAACTTTTGGTCAGGGCCAATAGA 1140
GTTACGTTTTGATTTTGTTCAAATGCGTTTCACACTGGAAGTGTGATTATATCAGCGGA 1200
GTATAATCGATCATCTACCAATACGGATGAGTGCCAATCTCACTCCACTTATACAAAAAC 1260
SBV vp1 sequence
GTTCCATTGGGAGAACAAAAATCCGTACATTTTACTGTGCCGTATATATATGATACCGT 1320
GTTACGTAGAAATACGGCTAGTGCCTATTTACCGTTACTGATAATGATAAGGTAGATAA 1380

TGTTAGTAAGGCGCAGGCTACGGGGATTAGAGCAGAGTCTAAAATGAGAGTGAAAGTGAG 1440
AGTAGTTAATGTTTTAAGGCCGTGTCCTCTACTACCTCAACTATAGAAGTTTGGTGTA 1500
TATGCGAGGAGGTACCATCTCGAGAGCACGCTGCAGTGCTTTCTTCATCTCCTTATCATT 1560
XhoI *RBS*
GATAAAAAATACTTCTCATTATATTATTTAAAACCTAAAAAGTTCATTAAATAACGTTCT 1620
AGACTTCTTATCATAATACATAATTTTCAAAGTATAAAATGATTTTTCATAAATCCAT 1680
TAGACGGTGCAAATATATGTTTTTAATGTTCTTCGTTTTTAGGCATCCCTCCTTCAAGA 1740
STAB-SD sequence
TTATCCCCGGGTACCGAGCTCAAATAAACAACCTCCTTAAGTTAATTAGAATAGTGGCCTT 1800
TCAACATTAAATTATGATTTTAAACTTGCAATTATTAATCAAATATAAAGTAACATAATA 1860
AATTAAATTGATAGACTTAATTCACATAATATTATTTAATGTATACATTGGTGCATGTTT 1920
AAGAATTAATTTATAAGGATAACGAATACTTTTTATCTAAATCCCCTCACACTAAAGTTT 1980
TTTTGTATACTTGAATATACCTCCTAAATTTTATTTGTTTTTGATTACCGTAGTATTCT 2040
Reverse *CytIAa* promoter
ATGCGCTATAGTTCGAAAGATGCCTGTTTTTTTAGTATGTATGCTCTTTTAAAGCAATAT 2100
ATATTTTGAATAAGTTTTAGAAATGGTATTGGGTTTTGAGTAATATATTTACAAATTGAT 2160
AGATTGAATTTAAAGACGCCCCTAATACTTCATAATAAACTAAAACATCTCTATTACCT 2220
TTCTCAGGCATTCAATCATATTTAAGTTTGAAAAATTTGAAATCGAAAAAGAAATCACT 2280
AGTTCTCATGCAAACTCAGGTTTAAATATCGTTTTCAAATCAATTGTCCAAGAGCAGCAT 2340
TACAAATAGATAAGTAATTTGTTGTAATGAAAAACGGACATCACCTCCATTGAAACGGAG 2400
Terminal sequence
TGATGTCCGTTTTACTATGTTATTTCTAGTAATACAGCATGC
SphI

Fig. 6. Nucleotide sequences of pHT1K-SBV vp1 vector cassette.

GTCGACTGTATTACTAGAAAATAACATAGTAAACGGACATCACTCCGTTTCAATGGAGG 60
SalI
TGATGTCCGTTTTTCATTACAACAAATTACTTATCTATTTGTAATGCTGCTCTTGGACAA 120
Reverse terminal sequence
TTGATTTGAAAACGATATTTAAACCTGAGTTTGCATGAGAACTAGTGAATTCTATTTTCG 180
ATTTCAAATTTTCCAAACTTAAATATGATTGAATGCCTGAGAAAGGTAATAGAGATGTTT 240
TAGTTTATTATGAAGTATTAGGGGCGTCTTTTAAATTCAATCTATCAATTTGTGAAATAT 300
ATTACTCAAACCCAATACCATTCTAAACTTATTCAAATATATATTGCTTTAAAGAG 360
CATACATACTAAAAAACAGGCATCTTTCGAACTATAGCGCATAGAATACTACGGTGAAT 420
***Cyt1Aa* promoter**
CAAAAACAAATAAAATTTAGGAGGTATATTCAAGTATACAAAAAACTTTAGTGTGAGGG 480
GATTTAGATAAAAAGTATTCGTTATCCTTATAAAATTAATTCTTAAACATGCACCAATGTA 540
TACATTAAATAATATTATGTGAATTAAGTCTATCAATTTAATTTATTATGTTACTTTATA 600
TTTGATTAATAATTGCAAGTTTAAATCATAATTTAATGTTGAAAGGCCACTATTCTAAT 660
TAACCTAAGGAGTTGTTTATTTGAGCTCGGTACCCGGGATAATCTTGAAAGGAGGGATG 720
STAB-SD sequence
CCTAAAAACGAAGAACATTAAAAACATATATTTGCACCGTCTAATGGATTATGAAAAAT 780
CATTTTATCAGTTTGAAAATTATGTATTATGATAAGAAAGTCTAGAACGTTATTTAATGA 840
ACTTTTtaggtttttaaataataataatgagaagtattttttatcaatgataaGGAGATGAA 900
RBS
GAAAGCACTCGAGAGCACGGGATCCATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGT 960
BamHI
GGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGG 1020
CGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGG 1080
CAAGCTGCCCCGTGCCCTGGCCACCCCTCGTGACCACCCCTGACCTACGGCGTGCAGTGCTT 1140
CAGCCGCTACCCCGACCACATGAAGCAGCAGGACTTCTTCAAGTCCGCCATGCCCGAAGG 1200
CTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGA 1260
EGFP sequence
GGTGAAGTTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAA 1320
GGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACAGCCACAACGTCTA 1380

TATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACAT 1440
CGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGG 1500
CCCCGTGCTGCTGCCCACAACCACTACATGGATCCTGCTTCTTCATCTCCTTATCATT 1560
*Bam**HI* RBS
GATAAAAAATACTTCTCATTATATTATTTAAAACCTAAAAAGTTCATTAAATAACGTTCT 1620
AGACTTCTTATCATAATACATAATTTTCAAAGTATAAAATGATTTTTCATAAATCCAT 1680
TAGACGGTGCAAATATATGTTTTTAATGTTCTTCGTTTTTAGGCATCCCTCCTTTCAAGA 1740
STAB-SD sequence
TTATCCCCGGGTACCGAGCTCAAATAAACAACTCCTTAAGTTAATTAGAATAGTGGCCTT 1800
TCAACATTAAATTATGATTTTAAACTTGCAATTATTAATCAAATATAAAGTAACATAATA 1860
AATTAAATTGATAGACTTAATTCACATAATATTATTTAATGTATACATTGGTGCATGTTT 1920
AAGAATTAATTTATAAGGATAACGAATACTTTTATCTAAATCCCCTCACACTAAAGTTT 1980
TTTGTATACTTGAATATACCTCCTAAATTTTATTTGTTTTGATTCACCGTAGTATTCT 2040
Reverse *Cyt**I*Aa promoter
ATGCGCTATAGTTCGAAAGATGCCTGTTTTTTTAGTATGTATGCTCTTTTAAAGCAATAT 2100
ATATTTTGAATAAGTTTGAATGGTATTGGGTTTTGAGTAATATATTTACAAATTGAT 2160
AGATTGAATTTAAAAGACGCCCCTAATACTTCATAATAAACTAAAACATCTCTATTACCT 2220
TTCTCAGGCATTCAATCATATTTAAGTTTGGAATAATTGAAATCGAAATAGAATTCACT 2280
AGTTCTCATGCAAACCTCAGGTTTAAATATCGTTTTTCAAATCAATTGTCCAAGAGCAGCAT 2340
TACAAATAGATAAGTAATTTGTTGTAATGAAAAACGGACATCACCTCCATTGAAACGGAG 2400
Terminal sequence
TGATGTCCGTTTTACTATGTTATTTTCTAGTAATACAGCATGC
*Sph**I*

Fig. 7. Nucleotide sequences of pHT1K-EGFP vector cassette.

4. Confirmation of transformed *B. thuringiensis* dsRNA

A. Confirmation of dsRNA by RT-PCR

To confirm the transcription of dsRNA from each strand, the cDNA of EGFP transgene was synthesized by using each forward and reverse primer, respectively. After that, reverse transcription PCR was performed as described previously. As this result, it was confirmed that both strands of dsRNA were amplified as expected by the 602 bp of PCR product (Fig. 9A).

B. Confirmation of dsRNA by qPCR

To figure out the relative transcription level of transformed *B. thuringiensis* transgene (EGFP), qPCR analysis was conducted. The RNA samples from the transformed *B. thuringiensis* were extracted at different cultivation times of 14, 18, 22, and 26 hours, and qPCR was performed by using EvaGreen 2X qPCR MasterMix (Applied Biological Materials Inc, Canada). A spore structure gene (*ssPE*) sequences of *B. thuringiensis* were used as reference. At 18 hours after cultivation point, the relative transcription level of transgene (EGFP) showed its highest level when the cell conditions were in sporulation stage and before autolysis begun (Fig. 9B, 9C).

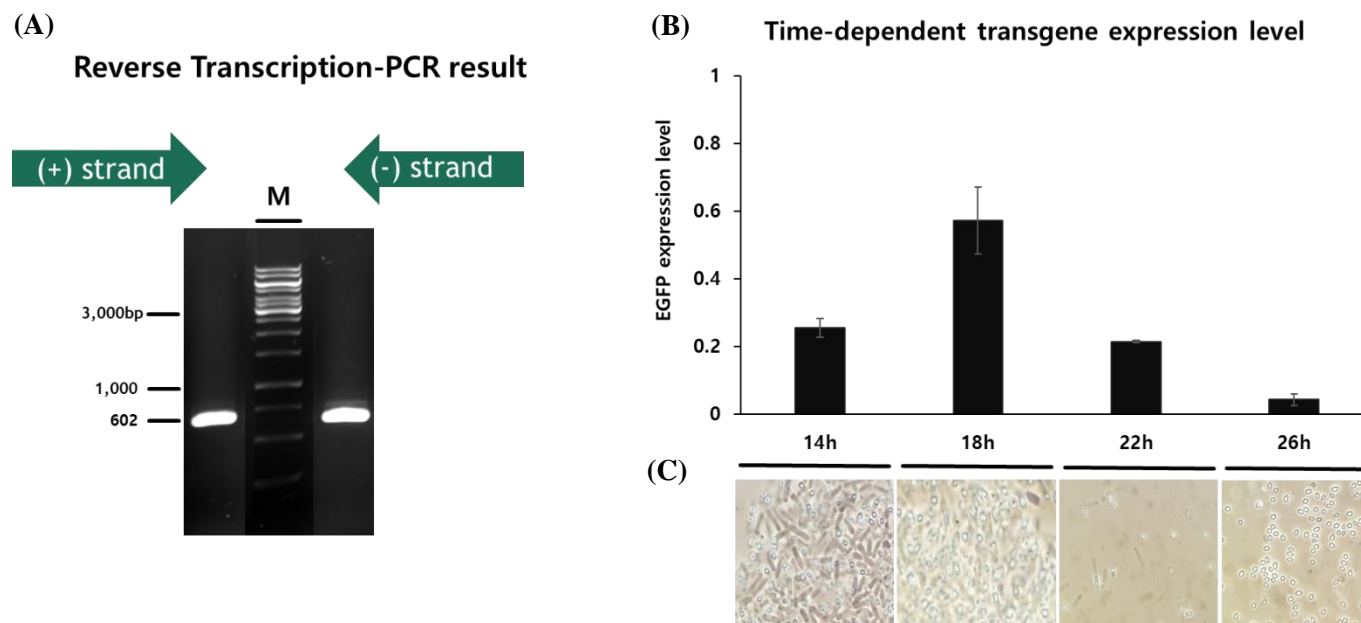


Fig. 9. Confirmation of dsRNA; RT-PCR & qPCR analysis. (A) Confirmation of transcription of EGFP gene from transformed *B. thuringiensis*. cDNAs were synthesized by using only (+): forward primer and (-): reverse primer respectively. (B) Expression profiles of EGFP transgene from transformed *B. thuringiensis*. (C) Phase contrast microscope data at different cultivation time point of transformed *B. thuringiensis*.

C. Confirmation of dsRNA by hybridization analysis

To detect the dsRNA of transgene (EGFP), hybridization assay of the total RNA of transformed *B. thuringiensis* was performed by using DIG System Users Guide (Roche Diagnostics) according to the manufacturer's instruction. Compared to the negative control which is the total RNA of *B. thuringiensis* israelensis 4Q7 strain, the pHT1K-EGFP transformed *B. thuringiensis* total RNA loaded lane showed bands (Fig. 10A). It was indicated that the EGFP dsRNA was produced by transformed *B. thuringiensis*. The 602 bp length of EGFP target gene PCR product after purified by QIAquick PCR Purification Kit (Qiagen) was used as positive control. As quantified dsRNA of EGFP with Molecular Imaging Software (Eastman Kodak Company, Rochester, NY) using the PCR amplified EGFP DNA. The amount of target dsRNA was 530 pg per 1 μ g of total RNA (Fig. 10B).

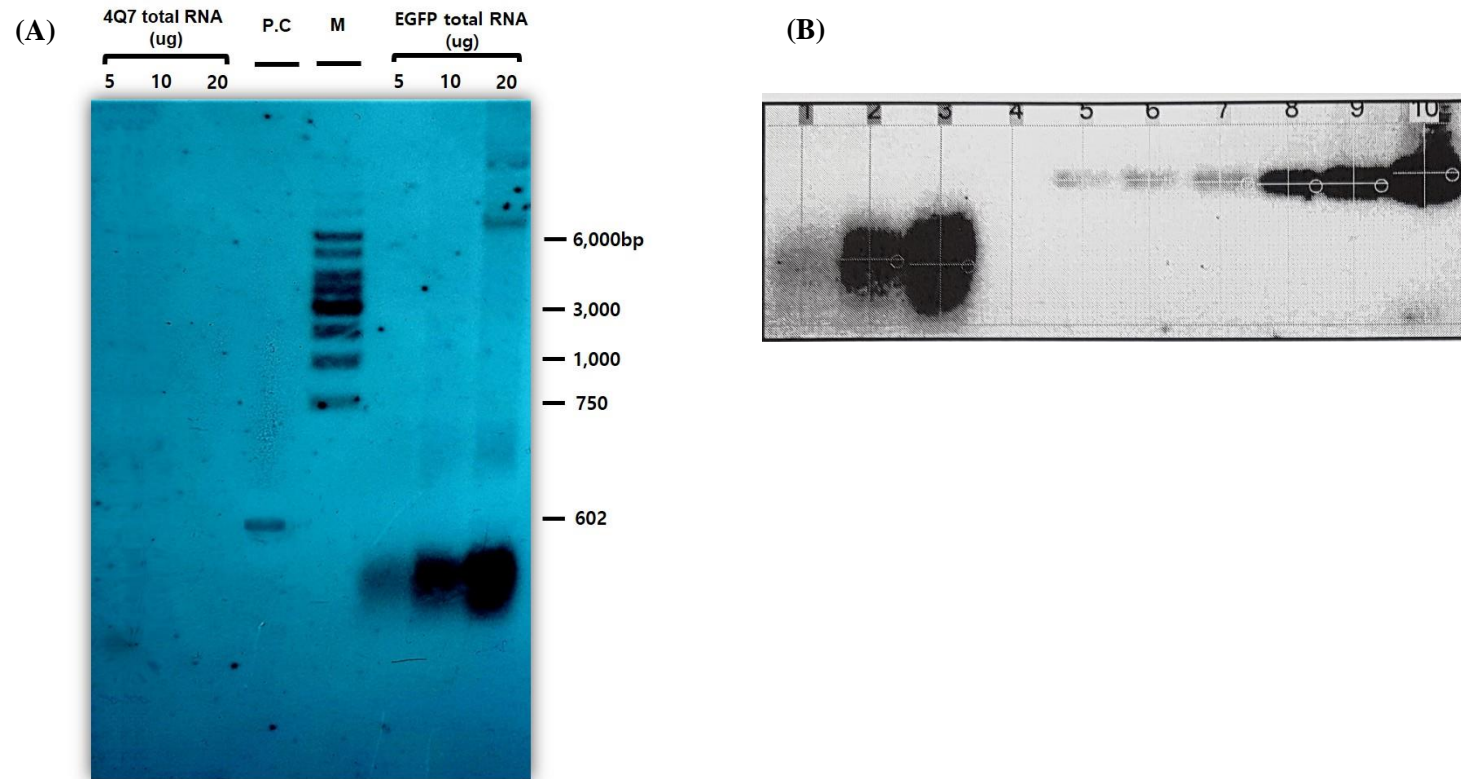


Fig. 10. Hybridization analysis of dsRNA synthesized by using transformed *B. thuringiensis*. (A) M: 1kbp ladder. P.C is positive control, which is PCR amplified 602 bp of EGFP DNA, and the total RNA of *B. thuringiensis* 4Q7 strain was used as negative control. (B) The quantification of EGFP dsRNA, which was 530 pg of dsRNA per 1 μ g of total RNA.

5. Optimization of *B. thuringiensis* autolysis conditions for bioassay

A. cerana bioassay samples containing dsRNA from the transformed *B. thuringiensis* was harvested by using the characteristics of autolysis, which is a process to release spore from sporulating cell. To find optimized condition for *B. thuringiensis* autolysis, the sporulating cells were harvested and resuspended in various kinds of buffers and incubated in different temperatures. As a result, the sporulating cells resuspended in PBS buffer for 48 hours at 37°C showed active autolysis, and the spores released into the medium was observed by phase contrast microscope (Fig. 11). It indicated that incubating condition in PBS buffer at 37°C is suitable for active autolysis of *B. thuringiensis* cells. In this study, honeybee bioassay samples from transformed *B. thuringiensis* were prepared in this condition.

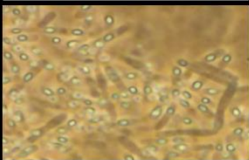
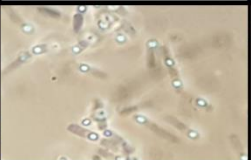
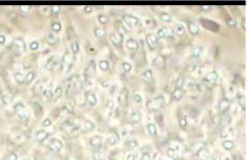
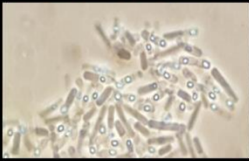
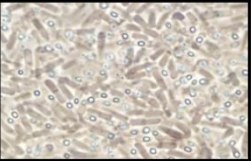
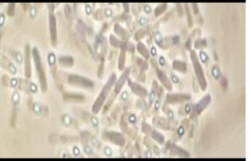
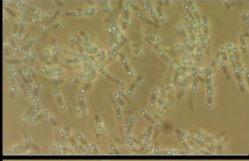
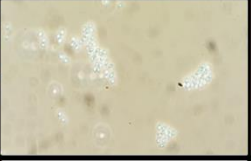
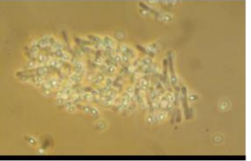
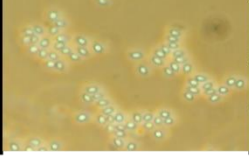
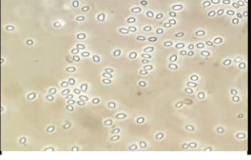
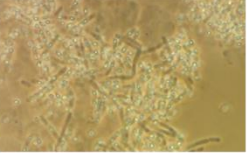
Medium (° C)	NaCl	PBS	2M MgCl ₂
4			
10			
25			
37			

Fig. 11. Optimization of autolysis conditions for bioassay. Transformed *B. thuringiensis* cells were resuspended according to various incubating temperature (4 °C, 10 °C, 25 °C, and 37 °C) and medium (NaCl, PBS, and 2M MgCl₂) to confirm best condition for autolysis.

6. *A. cerana* bioassay for transformed *B. thuringiensis* induced RNA interference

A. Concentration dependent honeybee safety test.

To confirm the safety of dsRNA samples isolated from the transformed *B. thuringiensis* for *A. cerana*, honeybees were fed with these dsRNA containing total RNA samples in various concentrations from 0.1 to 5 µg/µl for ten days. The mortality of the sample group that was fed with 5 µg/µl total RNA reached 20% of mortality in 3 days post treatment while the sample group that was fed with 3 µg/µl showed 15% of mortality in 10 days post treatment. The other sample groups fed with lower than 1.5 µg/µl showed only around 5% of mortality (Fig. 12).

B. Detection of SBV gene knockdown by feeding of dsRNA from pHT1K-SBV vp1 transformed *B. thuringiensis*

Experiments were carried out to examine whether feeding of total RNA containing dsRNA from pHT1K-SBV vp1 transformed *B. thuringiensis* would protect bees from SBV infection. To confirm the target gene knockdown, qPCR was conducted using dsRNA-treated *A. cerana*. As a result, the 1.5 µg/µl of total RNA containing dsRNA from pHT1K-SBV vp1 transformed *B. thuringiensis* treated group showed the effects of on the suppression of SBV replication as low as the negative control which treated with 40% (w/v) sucrose solution only (Fig. 13A).

**C. Concentration dependent suppression of SBV by dsRNA from pHT1K-SBV
vp1 transformed *B.thuringiensis***

To confirm the concentration dependent target gene knockdown by dsRNA from pHT1K-SBV vp1 transformed *B. thuringiensis*, qPCR was conducted using honeybees treated with various concentrations of dsRNA. The efficiency of suppression on SBV replication by dsRNA was significant at 1.0 µg/µl fed group and 1.5 µg/µl showed the highest efficiency. The tendency of transcriptional suppression effect of dsRNA from pHT1K-SBV vp1 transformed *B. thuringiensis*, treatment was proportionally related to the concentration of the dsRNA (Fig. 13B).

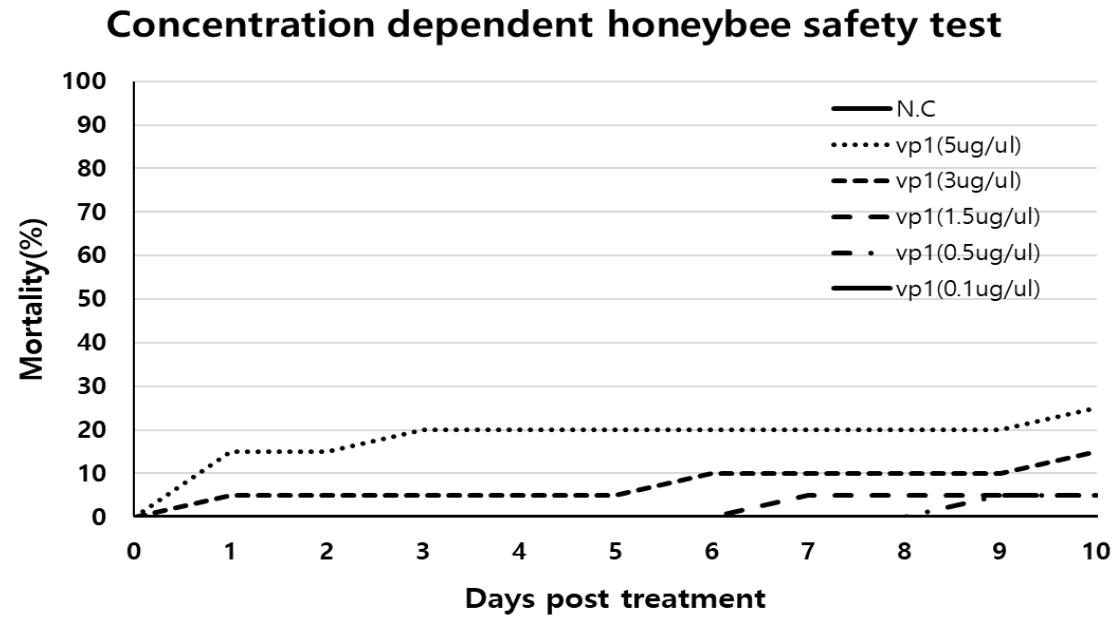


Fig. 12. pHT1K-SBV vp1 vector transformed *B. thuringiensis* total RNA concentration dependent honeybee safety test. (N.C; Negative control: feeding 40% (w/v) sucrose solution only.) The mortality of the worker bees fed with various concentration of dsRNA containing total RNA solutions was recorded for ten days.

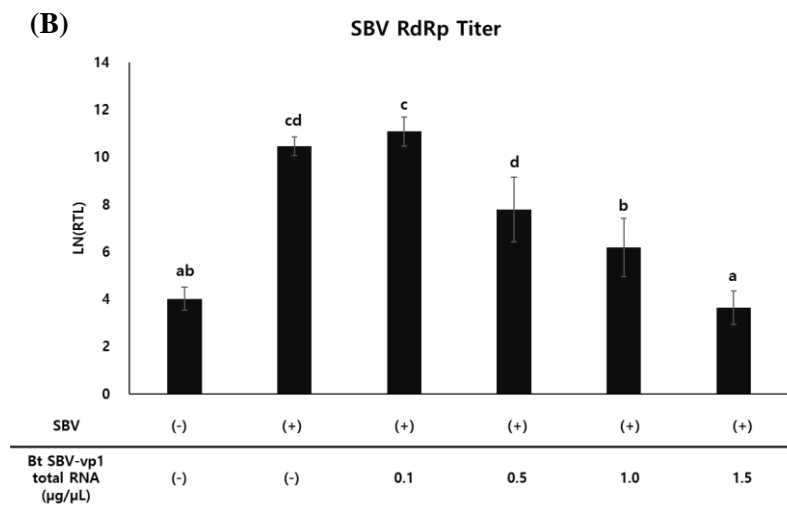
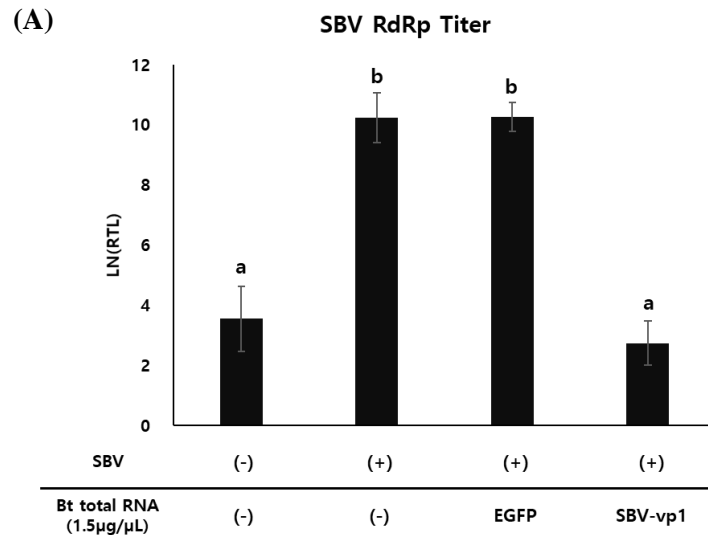


Fig. 13. *Apis cerana* bioassay with total RNA of transformed *Bacillus thuringiensis*. Each sample groups were infected with SBV (10^9 virus/mL) for 12h, and treated with dsRNA containing total RNA (A) 1.5 µg/µl, (B) concentration dependent (0.1 to 1.5 µg/µl) relative virus replication levels of total RNA treated workers.

DISCUSSION

RNA interference (RNAi) is a post-transcriptional gene regulation mechanism found in virtually all plants and animals including insects. Using dsRNA ingestion triggered gene silencing is a powerful technology for manipulating gene expression and can serve as a novel approach for controlling agriculture pests and potential therapeutic strategy for treating and preventing of diseases in beneficial insects for crops. Since small dsRNAs are required for various RNAi applications, there is a need for cost-effective methods for producing large quantities of high-quality dsRNA.

In this study, to produce exogenous dsRNA through simple and cost-effective methods, there is a focus on developing entomopathogenic bacteria *Bacillus thuringiensis*.

For the first step was to find a target that confirming dsRNA triggered gene silencing phenomenon such as ‘RNAi system in Sacbrood virus’. Sacbrood virus (SBV) in *Apis cerana* is prevalent in Asia, especially in Korea. It has been seriously endangered which caused over 75% serious colony loss and destroying honeybee industry. Hence developing the methods for the control of this Sacbrood disease is urgently needed. In previous study, there were attempting to protect honey bees from viruses by antiviral RNAi systems such as IAPV (Maori et al., 2009), and Deformed wing virus (Desai et al., 2012). The present study demonstrated that SBV transcripts was silenced in *A.cerana* by feeding of segments of SBV derived dsRNA including siRNA putative candidates such as dsVP1, dsVP3, and dsRdRp. It is also indicated that an RNAi system pathway of silencing suppressed Sacbrood virus replication. In further study, RNAi method to suppress SBV replication in worker

bees, may also use in the field protecting from SBV infection.

The capsid proteins of SBV contain four kinds of structural proteins (Zhang et al., 2002). In this study, it is found that all of dsRNA corresponding to VP1 gene (dsVP1), VP3 gene (dsVP3), and RdRp gene (dsRdRp) respectively, could silence the corresponding genes of SBV. However, there is no synergistic effect among these dsRNA. This indicates that the SBV polyprotein may also have the characteristic functional motifs attributed to helicases or proteases of viruses (Koonin et al., 1993). If one of the genes was silenced, SBV may not replicate in the bees. Likewise, replication and infection of West Nile virus, a positive single stranded RNA virus, was greatly reduced when siRNA was introduced by cytoplasmic-targeted transfection prior to but not after the establishment of viral replication (Geiss et al., 2005). In this research, dsVP1, dsVP3, and dsRdRp took RNAi effects in the SBV infected *Apis cerana* workers, as fed with these dsRNAs and confirmed that among the three dsRNAs, *vp1* gene specific dsRNA (dsVP1) showed a powerful effect of reducing SBV replication (Fig. 3, 4).

For the second step, it is necessary to using the characteristics of *B.thuringiensis* and applicates to set up a vector design strategy. In this study, the plasmid vectors, pHT1K-SBV *vp1* and pHT1K-EGFP, for bidirectional transcription of the desired dsRNA were designed. These vectors have the following three properties. First, these carry two origins of replication and two selection markers: the pUC replication sequence and ampicillin resistant gene for amplification and selection in *E.coli*; the Bt origin and erythromycin resistance gene, for amplification and selection in *B.thuringiensis* (Fig. 8). Second, these vectors have two bidirectional sporulation dependent *Cyt1Aa* promoters. Third, these vectors have two bidirectional STAB-SD sequence and RBS sites to increase stability of

target dsRNA in transcriptional level (Agaisse and Lereclus, 1996; Park et al., 1998).

The yield of interfering RNA could be improved if degradation of dsRNA in the bacterial host could be minimized. By removing double-strand-specific RNase from the bacterial cell, it might be accomplished. A dsRNA specific endonuclease (RNaseIII) is encoded by the bacterial *rnc* gene, and mutations in this gene are available in *E.coli* and *Bacillus subtilis* (Herskovitz and Bechhofer, 2000; Timmons et al., 2001). RNaseIII plays a major role in the processing and degradation of a subset of bacterial RNAs (Crouch, 1974; Dasgupta et al., 1998; Takiff et al., 1989). An important role of bacterial RNaseIII is to initiate the maturation of rRNA from the 30S precursor RNA (Bram et al., 1980; Sirdeshmukh and Schlessinger, 1985). Furthermore, RNaseIII has both side of positive and negative effects on the translational efficiency of specific mRNAs. It is by inducing conformational alterations through site-specific cleavages, by altering the stability of certain mRNAs, and by removing sequences that stimulate or inhibit ribosome binding (Hagen and Young, 1978; Portier et al., 1987; Takata et al., 1987). Despite the relative importance of the reactions catalyzed by RNaseIII, null mutations in *rnc* are compatible with growth (Takiff et al., 1989). In further study, it is necessary to developing a *B.thuringiensis* strain that is useable for RNAi, which dsRNA could be expressed without disturbing by host RNaseIII. Ultimately, RNaseIII-deficient strain of *B.thuringiensis* may significantly improves the efficiency of RNAi by feeding, likely because the dsRNA fragments produced are more stable in the bacteria.

LITERATURES CITED

- A Federici, B., Park, H.-W., Bideshi, D., 2010. Overview of the Basic Biology of *Bacillus thuringiensis* with Emphasis on Genetic Engineering of Bacterial Larvicides for Mosquito Control.
- Agaisse, H., Lereclus, D., 1996. STAB-SD: a Shine–Dalgarno sequence in the 5' untranslated region is a determinant of mRNA stability. *Mol. Microbiol.* 20, 633–643.
- Agaisse, H., Lereclus, D., 1995. How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? *J. Bacteriol.* 177, 6027–6032.
- Allen, M., Ball, B., 1996. The incidence and world distribution of honey bee viruses. *Bee World* 77, 141–162.
- Bailey, L., 1969. The multiplication and spread of sacbrood virus of bees. *Ann. Appl. Biol.* 63, 483–491.
- Bailey, L., Fernando, E.F.W., 1972. Effects of sacbrood virus on adult honey-bees. *Ann. Appl. Biol.* 72, 27–35.
- Bailey, L., Gibbs, A.J., Woods, R.D., 1964. Sacbrood virus of the larval honey bee (*Apis mellifera* Linnaeus). *Virology* 23, 425–429. [https://doi.org/10.1016/0042-6822\(64\)90266-1](https://doi.org/10.1016/0042-6822(64)90266-1)
- Baum, J.A., Malvar, T., 1995. Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. *Mol. Microbiol.* 18, 1–12.
- Bram, R.J., Young, R.A., Steitz, J.A., 1980. The ribonuclease III site flanking 23S

- sequences in the 30S ribosomal precursor RNA of *E. coli*. *Cell* 19, 393–401.
- Charles P. Moran, J., 1993. RNA Polymerase and Transcription Factors 653–667.
- Choe, S.-E., Nguyen, T.T.-D., Hyun, B.-H., Noh, J.-H., Lee, H.-S., Lee, C.-H., Kang, S.-W., 2012. Genetic and phylogenetic analysis of South Korean sacbrood virus isolates from infected honey bees (*Apis cerana*). *Vet. Microbiol.* 157, 32–40.
- Consortium, T.H.G.S., 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* 443, 931.
- Crouch, R.J., 1974. Ribonuclease III Does Not Degrade Deoxyribonucleic Acid-Ribonucleic Acid Hybrids. *J. Biol. Chem.* 249, 1314–1316.
- Dasgupta, S., Fernandez, L., Kameyama, L., Inada, T., Nakamura, Y., Pappas, A., Court, D.L., 1998. Genetic uncoupling of the dsRNA-binding and RNA cleavage activities of the Escherichia coli endoribonuclease RNase III — the effect of dsRNA binding on gene expression. *Mol. Microbiol.* 28, 629–640.
- Desai, S.D., Eu, Y.-J., Whyard, S., Currie, R.W., 2012. Reduction in deformed wing virus infection in larval and adult honey bees (*Apis mellifera* L.) by double-stranded RNA ingestion. *Insect Mol. Biol.* 21, 446–455.
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806.
- Geiss, B.J., Pierson, T.C., Diamond, M.S., 2005. Actively replicating West Nile virus is resistant to cytoplasmic delivery of siRNA. *Viol. J.* 2, 53.
- Geley, S., Müller, C., 2004. RNAi: ancient mechanism with a promising future. *Exp. Gerontol.* 39, 985–998.

- Glatron, M.F., Rapoport, G., 1972. Biosynthesis of the parasporal inclusion of *Bacillus thuringiensis*: half-life of its corresponding messenger RNA. *Biochimie* 54, 1291–1301.
- Hagen, F.S., Young, E.T., 1978. Effect of RNase III on efficiency of translation of bacteriophage T7 lysozyme mRNA. *J. Virol.* 26, 793–804.
- Hannon, G.J., 2002. RNA interference [WWW Document]. *Nature*.
- Herskovitz, M.A., Bechhofer, D.H., 2000. Endoribonuclease RNase III is essential in *Bacillus subtilis*. *Mol. Microbiol.* 38, 1027–1033.
- Höfte, H., Whiteley, H.R., 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* 53, 242–255.
- Koonin, E.V., Dolja, V.V., Morris, D.T.J., 1993. Evolution and Taxonomy of Positive-Strand RNA Viruses: Implications of Comparative Analysis of Amino Acid Sequences. *Crit. Rev. Biochem. Mol. Biol.* 28, 375–430.
- Koressaar, T., Remm, M., 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23, 1289–1291.
- Lanzi, G., Miranda, J.R. de, Boniotti, M.B., Cameron, C.E., Lavazza, A., Capucci, L., Camazine, S.M., Rossi, C., 2006. Molecular and Biological Characterization of Deformed Wing Virus of Honeybees (*Apis mellifera* L.). *J. Virol.* 80, 4998–5009.
- Lereclus, D., Arantès, O., Chaufaux, J., Lecadet, M.-M., 1989. Transformation and expression of a cloned δ -endotoxin gene in *bacillus thuringiensis*. *FEMS Microbiol. Lett.* 60, 211–217.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* 25, 402–408.

- Maori, E., Paldi, N., Shafir, S., Kalev, H., Tsur, E., Glick, E., Sela, I., 2009. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. *Insect Mol. Biol.* 18, 55–60.
- Miranda, J.R. de, Bailey, L., Ball, B.V., Blanchard, P., Budge, G.E., Chejanovsky, N., Chen, Y.-P., Gauthier, L., Genersch, E., Graaf, D.C. de, Ribière, M., Ryabov, E., Smet, L.D., Steen, J.J.M. van der, 2013. Standard methods for virus research in *Apis mellifera*. *J. Apic. Res.* 52, 1–56.
- Park, H.-W., Ge, B., Bauer, L.S., Federici, B.A., 1998. Optimization of Cry3A Yields in *Bacillus thuringiensis* by Use of Sporulation-Dependent Promoters in Combination with the STAB-SD mRNA Sequence. *Appl. Environ. Microbiol.* 64, 3932–3938.
- Peng, Y.-S., Fang, Y., Xu, S., Ge, L., 1987. The resistance mechanism of the Asian honey bee, *Apis cerana* Fabr., to an ectoparasitic mite, *Varroa jacobsoni* Oudemans. *J. Invertebr. Pathol.* 49, 54–60.
- Portier, C., Dondon, L., Grunberg-Manago, M., Régnier, P., 1987. The first step in the functional inactivation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5' end. *EMBO J.* 6, 2165–2170.
- Rasko, D.A., Altherr, M.R., Han, C.S., Ravel, J., 2005. Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol. Rev.* 29, 303–329.
- Rosenkranz, P., Aumeier, P., Ziegelmann, B., 2010. Biology and control of *Varroa destructor*. *J. Invertebr. Pathol.* 103, S96–S119.
<https://doi.org/10.1016/j.jip.2009.07.016>
- Schmid-Hempel, R., Schmid-Hempel, P., 1998. Colony performance and

- immunocompetence of a social insect, *Bombus terrestris*, in poor and variable environments. *Funct. Ecol.* 12, 22–30. <https://doi.org/10.1046/j.1365-2435.1998.00153.x>
- Sirdeshmukh, R., Schlessinger, D., 1985. Ordered processing of *Escherichia coli* 23S rRNA in vitro. *Nucleic Acids Res.* 13, 5041–5054. <https://doi.org/10.1093/nar/13.14.5041>
- Takata, R., Mukai, T., Hori, K., 1987. RNA processing by RNase III is involved in the synthesis of *Escherichia coli* polynucleotide phosphorylase. *Mol. Gen. Genet.* MGG 209, 28–32. <https://doi.org/10.1007/BF00329832>
- Takiff, H.E., Chen, S.M., Court, D.L., 1989. Genetic analysis of the *rnc* operon of *Escherichia coli*. *J. Bacteriol.* 171, 2581–2590. <https://doi.org/10.1128/jb.171.5.2581-2590.1989>
- Timmons, L., Court, D.L., Fire, A., 2001. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* 263, 103–112. [https://doi.org/10.1016/S0378-1119\(00\)00579-5](https://doi.org/10.1016/S0378-1119(00)00579-5)
- Waterhouse, P.M., Wang, M.-B., Lough, T., 2001. Gene silencing as an adaptive defence against viruses [WWW Document]. *Nature*. <https://doi.org/10.1038/35081168>
- Wong, H.C., Schnepf, H.E., Whiteley, H.R., 1983. Transcriptional and translational start sites for the *Bacillus thuringiensis* crystal protein gene. *J. Biol. Chem.* 258, 1960–1967.
- Zhang, Q., Yang, Y., Liang, Y., Lu, X., Zhang, J., 2002. Study on structure of the Chinese Sacbrood Virus nucleic acid. *J. Chin. Electron Microsc. Soc.* 21, 331–334.

ABSTRACT ON KOREAN

Bacillus thuringiensis 기반의 외인성 이중가닥

RNA 생산 플랫폼 구축

서울대학교

농생명공학부 곤충학전공

박 민 구

초 록

RNA 간섭 (RNA interference)은 이중가닥 RNA (dsRNA)를 이용한 유전자 발현 억제 기술로서 식물과 곤충을 포함한 동물에서 적용 가능하다. 이중가닥 RNA에 의해 일련의 RNA 간섭 과정을 거쳐 특정한 상동 mRNA를 선택적으로 분해하여 유전의 발현을 조절하기에 해충방제 및 유용 곤충의 질병 치료에 해당하는 농업, 인간의 질병 치료인 의학 등 다양한 분야에서 이용되고 있다. RNAi 기술이 이렇게 다양한 분야에 활용되기 때문에 dsRNA의 간단하고 경제적인 대량생산 기술이 요구된다. 현재까지 dsRNA를 합성하는 본래의 기술

은 크게 바이러스를 이용한 방법, 형질전환 세균을 이용한 방법, 형질전환 작물을 이용한 방법이 있다. 본 연구에서는 곤충병원성 미생물인 *Bacillus thuringiensis*를 기반으로 dsRNA 생산 플랫폼을 개발하는 것에 주목했다.

첫번째로, dsRNA가 RNA 간섭 과정속에서 제대로 작동 하는지 확인하기 위하여, 국내에서 심각한 문제가 되었던 토종벌(*Apis cerana*)의 낭충봉아부패병(Sacbrood virus)의 바이러스 증식을 억제하는 dsVP1, dsVP3, dsRdRp 세가지의 dsRNA들을 합성(in vitro)하여 낭충봉아부패병 바이러스에 감염된 토종벌 일벌에게 먹였다. 그 결과, 모든 dsRNA에서 바이러스 증식이 억제되는 것을 확인하였고, 그 중 vp1 유전자를 목적으로 한 dsVP1의 효과가 가장 강력한 것으로 나타났다.

두번째로, *B. thuringiensis* 기반의 dsRNA의 생산을 위해 균주의 특성을 이용하는 plasmid 벡터를 디자인하였다. 여기에는 *B. thuringiensis*의 Cyt 독소 생산에 사용하는 강력한 cyt1Aa 프로모터와, 목적 유전자를 과발현 및 안정화 시켜주는 STAB-SD 시퀀스를 포함하여 유전자 전사 수준에서 dsRNA의 과발현을 유도하였다. 제작이 완료된 pHT1K-SBV vp1과 pHT1K-EGFP plasmid 벡터를 곤충병원성 미생물인 *B. thuringiensis israelensis* 4Q7 균주에 형질전환 시켰다. 역전사 중합효소 연쇄반응, 실시간 중합효소 연쇄반응 및 hybridization assay를 통하여 dsRNA의 전사를 확인하였다.

마지막으로 합성된 dsRNA에 의하여 RNA 간섭 작용이 가능한지를 검증하기 위하여 토종벌의 낭충봉아부패병 실험을 하였다. 낭충봉아부패병 바이러스

에 감염된 토종벌 일벌에게 pHT1K-SBV *vp1* 형질전환 *B. thuringiensis*에서 나온 dsRNA를 먹인 후 실시간 중합효소 연쇄반응을 통하여 확인한 결과, 바이러스의 증식 억제효과가 매우 뛰어난 것을 확인하였다.

본 연구를 통하여, 곤충병원성 미생물인 *B. thuringiensis* 기반의 외인성 이중가닥 RNA 생산 플랫폼의 가능성을 확인하였다. 또한, 실험을 통해 찾아낸 토종벌 낭충봉아부패병의 증식을 억제하는 효과적인 *vp1* 유전자를 *B. thuringiensis* 기반의 생산 플랫폼에 적용하여 얻은 dsRNA는 매우 강력한 바이러스 증식 억제 효과를 가지고 있다. 추후에, 이 플랫폼을 통하여 생산된 dsRNA는 낭충봉아부패병 억제를 비롯하여 RNA 간섭을 이용하는 다른 여러 가지 분야에도 유용할 것으로 판단되었다.

Key words: RNA 간섭, 유전자 침묵, 이중가닥 RNA, 토종벌, 낭충봉아부패병

학번: 2016-21725